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STUDIES ON THE CATALYTIC
MECHANISM OF PIG-PLASMA
BENZYLAMINE OXIDASE

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BY

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This thesis is based upon the following publications.

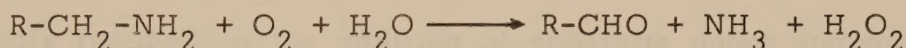
1. Active-site titration of pig-plasma benzylamine oxidase with hydrazine derivatives.
Lindström, A. and Pettersson, G., Eur. J. Biochem. 34, 564-568 (1973).
2. The mechanism of inhibition of pig-plasma benzylamine oxidase by the copper-chelating reagent cuprizone.
Lindström, A. and Pettersson, G., Eur. J. Biochem. in press.
3. Effect of azide on some spectral and kinetic properties of pig-plasma benzylamine oxidase.
Lindström, A., Olsson, B. and Pettersson, G., Eur. J. Biochem. in press.
4. Transient kinetics of benzaldehyde formation during the catalytic action of pig-plasma benzylamine oxidase.
Lindström, A., Olsson, B. and Pettersson, G., Eur. J. Biochem. 42, 377-381 (1974).
5. Kinetics of the interaction between pig-plasma benzylamine oxidase and substrate.
Lindström, A., Olsson, B. and Pettersson, G. Eur. J. Biochem. 35, 70-77 (1973).

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INTRODUCTION

Amines are of fundamental importance in living organisms, both as structural components of proteins and as substrates for many metabolic processes. A number of amines also play important roles in the regulation of many cell activities, as neurotransmitters and in other ways. The biosynthesis of amines usually proceeds via decarboxylation of amino acids; the catabolism of biogenic amines often involves oxidative deamination by a group of enzymes called amine oxidases. These oxidases degrade a variety of mono- and diamines with the formation of the corresponding aldehyde and ammonia. Concomitantly, two electrons are transferred from the amine to molecular oxygen, which is reduced to hydrogen peroxide:



The detailed mechanism of this enzymatically catalyzed electron transfer process is not well understood.

Amine oxidase activity has been found in various tissues of animals and plants and in microorganisms. Early attempts to classify the amine oxidases were based upon their substrate specificity (monoamine oxidases vs. diamine oxidases), but this classification has proven unsatisfactory since some amine oxidases act upon both mono and diamines. A more fruitful approach is to differentiate these enzymes according to their inhibitor specificity. Two main groups of amine oxidases can be distinguished, those inhibited by carbonyl reagents and those unaffected by carbonyl reagents (1). Enzymes of the latter group, such as the classical monoamine oxidase, are usually located in

mitochondria and have in several cases been found to contain FAD as a prosthetic group. The amine oxidases sensitive to carbonyl reagents include the classical diamine oxidase, and enzymes such as spermine oxidase and benzylamine oxidase, all of which have been reported to contain firmly bound copper and pyridoxal phosphate. Although the copper-pyridoxal-containing amine oxidases appear to be closely related, they exhibit striking dissimilarities in several physico-chemical respects and should preferably, for the time being, be considered as distinctly separate enzymes. The present thesis is concerned with the amine oxidase isolated from pig plasma, usually referred to as benzylamine oxidase.

Benzylamine oxidase has a molecular weight of 195 000 (2). Its optical absorption spectrum shows a sharp maximum at 280 nm and a broad and fairly weak absorption band at 470 nm. The 470 nm absorbance band is significantly reduced on the addition of substrate under anaerobic conditions, but can be fully restored by aeration of the reaction solution. Benzylamine oxidase has been reported to contain 3-4 moles of pyridoxal phosphate per mole protein (3). The copper content appears to be 2 atoms per protein molecule (4). The enzyme is strongly inhibited by carbonyl reagents such as hydrazine derivatives, and by the copper-chelating reagent cuprizone (biscyclohexanone oxalyldihydrazone). Benzylamine is usually used as a standard substrate for the enzyme, but a variety of ring-substituted benzylamine derivatives (5) and biogenic amines such as tyramine, tryptamine and histamine (2) are also oxidised. The biological function of benzylamine oxidase has not yet been established.

The investigations described in the present thesis have been directed towards the elucidation of the reaction mechanism of benzylamine oxidase, with particular reference to the catalytic functions of copper and pyridoxal phosphate.

This problem is of interest both because benzylamine oxidase is a typical member of the copper-pyridoxal-containing group of amine oxidases, and because information obtained about this enzyme should contribute to current efforts to obtain a detailed knowledge of the structure and function of pyridoxal enzymes and copper proteins in general.

PYRIDOXAL PHOSPHATE IN BENZYLAMINE OXIDASE

Schiff base formation between substrates and pyridoxal phosphate has been suggested as the rate-limiting step in oxidative deaminations by plasma amine oxidases (6). Attempts made to stabilize the intermediate Schiff base by borohydride reduction have led to the conclusion that 3 moles of substrate are bound per mole of benzylamine oxidase (7). The content of pyridoxal phosphate in benzylamine oxidase is 3-4 moles per mole enzyme according to microbiological assays and phosphorus analyses (3). This suggests that there are probably 3-4 active sites per enzyme molecule. However, the protein appears to contain only two copper atoms, which, especially in view of the 1:1 correspondence between copper and pyridoxal phosphate in the closely related diamine oxidase from pig kidney (16), indicates the presence of two active sites in benzylamine oxidase.

In order to obtain more direct evidence concerning the number of catalytically reactive carbonyl groups in benzylamine oxidase, the enzyme was submitted to active-site titration with hydrazine derivatives (Paper 1). Treatment of benzylamine oxidase with an excess of phenylhydrazine leads to the appearance of a new absorption band centered around 430 nm. The reaction cannot be reversed by dialysis and permanently deactivates the enzyme.

The amount of inhibitor required for maximum increase of the 430 nm absorbance corresponded to one mole per mole of enzyme, and this gave complete inactivation. A preliminary examination of the kinetics of the inactivation process indicated that the hydrazine combines irreversibly with the enzyme in competition with the substrate. Thus, it appears that the pyridoxal phosphate groups that react with carbonyl reagents are identical with those required for enzymatic activity and that the enzyme contains only one such group. A more detailed stopped-flow spectrophotometric examination of the

kinetics of the interaction between benzylamine oxidase and various hydrazine derivatives (8) provided strong supporting evidence that substrates and hydrazines compete for the reactive carbonyl group in the enzyme, confirming that Schiff base formation between pyridoxal phosphate and the amine substrate may be of primary importance in the catalytic mechanism. The inhibitory action of cuprizone on benzylamine oxidase and related enzymes has been generally assumed to be due to the copper-chelating properties of this reagent. Paper 2 however provides evidence that the inhibitory effect of cuprizone is due to an interaction with protein-bound pyridoxal phosphate rather than with protein-bound copper. Addition of cuprizone to the enzyme leads to the appearance of a new absorption band at 350 nm, similar to the absorption band appearing when cuprizone was added to free pyridoxal phosphate. Furthermore, cuprizone interacts with the enzyme in strict competition with phenylhydrazine, and substrates protect the enzyme against inhibition by cuprizone in an apparently competitive manner. It seems likely that cuprizone, being a hydrazine, might undergo a transimination reaction with the protein-bound pyridoxal phosphate, leading to inhibition of enzyme activity by reaction with the catalytically essential carbonyl group. Spectrophotometric titration of the enzyme established that one mole of cuprizone was sufficient to complete the inactivation of the enzyme and the change in the 350 nm absorbance. This provides further confirmation of the presence of a single active site in benzylamine oxidase.

COPPER IN BENZYLAMINE OXIDASE

The role of copper in copper-pyridoxal-containing amine oxidases has not been clarified and there is conflicting evidence as to whether or not copper goes through a reduction-oxidation cycle during the catalytic process. Indications that a valence change of copper may occur during catalysis have been

obtained from stopped-flow kinetic experiments with pig kidney diamine oxidase (9). However; no evidence of any copper valence change was obtained on application of similar kinetic techniques to benzylamine oxidase (Paper 5) and there was no EPR-detactable valence change on the addition of substrate to benzylamine oxidase under anaerobic conditions or in the steady-state reaction. This does not exclude a reduction of copper as an obligatory step in the catalytic mechanism, since a rapid redox equilibration between several components in the enzymatic system could favour restoration of the copper to the oxidized state and result in a less than stoichiometric reduction of the EPR signal by substrate under anaerobic conditions (9).

The observation that cuprizone acts as a strong inhibitor of various plasma amine oxidases has been taken to indicate that copper is essential for the catalytic activity of these proteins (10). As was mentioned above however the inhibition of benzylamine oxidase by cuprizone appears to be due to an interaction with pyridoxal phosphate rather than with copper (Paper 2). No extensive EPR spectral changes were observed after reaction of the enzyme with stoichiometric concentrations of cuprizone even though the enzyme activity was almost completely abolished by this treatment. In the presence of increasing concentrations of free cuprizone, the EPR spectrum of benzylamine oxidase changed significantly in a way suggesting a reversible interaction between cuprizone and protein-bound copper. The corresponding binding constant did not agree in magnitude with that calculated kinetically for the inhibitory interaction between cuprizone and the enzyme.

By analogy with other copper-containing oxidases, one might expect that a main role of copper in benzylamine oxidase is to facilitate the reduction of oxygen. Direct evidence for this view is however lacking, and it is noteworthy that azide has been reported not to inhibit copper-pyridoxal-containing

amine oxidases (17-19); azide usually binds strongly to copper and it is a most efficient inhibitor of copper-containing oxidases such as laccase and ceruloplasmin (11-13). The apparent lack of effect of azide on benzylamine oxidase could indicate either that copper is not accessible to ligands in solution or that ligand-binding to copper does not necessarily have any noticeable effect on enzyme activity. The results with cuprizone suggested that the latter explanation might be valid, and in order to discriminate more clearly between the two possibilities the effect of azide on EPR spectral properties of benzylamine oxidase was investigated (Paper 3).

It was found that azide has a very significant effect on the EPR signal of benzylamine oxidase. Apparently it binds directly to both copper ions in the protein with a binding constant of 0.1-0.2 mM. The affinity of azide for the protein was not significantly affected by reduction of the enzyme with substrate, or by formation of the phenylhydrazone derivative of the protein. Azide at concentrations below 1 mM has no effect on the steady-state or transient-state kinetics of the catalytic process. These observations provide evidence that the enzyme-bound copper is accessible to external ligands, and strongly indicate that ligand-binding to copper need not necessarily interfere with the catalytic process.

While the above results obtained with low concentrations of azide might seem to suggest that copper has no direct catalytic function in benzylamine oxidase, examination of the effect of high concentrations of azide on the enzyme provided evidence in the opposite direction (Paper 3). A reversible binding of azide to copper in the protein, corresponding to a binding-constant of about 40 mM, can be inferred from the azide-induced changes in the EPR and optical spectra of the protein. This enzyme-azide complex has an absorption band at 390 nm, similar to that appearing when azide binds to Type 2 copper

in ceruloplasmin (13). Formation of the chromophoric enzyme-azide complex was found to parallel an inhibition of enzyme activity. The inhibitory effect of high concentrations of azide could not be related to any decreased reactivity of the protein-bound pyridoxal phosphate, but there are strong indications from kinetic data that azide interferes with the reoxidation of the reduced enzyme by oxygen. It appears that at least one of the copper ions in benzylamine oxidase has a catalytically important function, and this function may be related to the process of electron-transfer from the substrate-reduced enzyme to molecular oxygen.

REACTION MECHANISM OF BENZYLAMINE OXIDASE

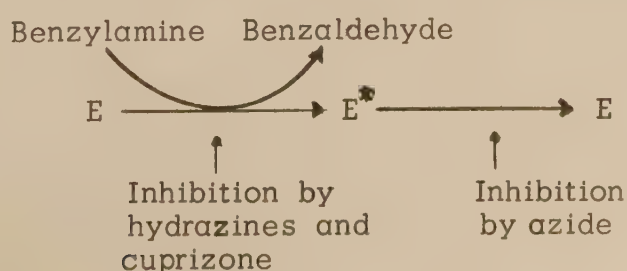
Evidence has been obtained that benzylamine oxidase operates via a ping-pong type of mechanism (14,15) but the order of product release is not wholly clarified. From product inhibition studies and the observation that no benzaldehyde was formed on mixing enzyme with benzylamine anaerobically, Taylor et. al. suggested a scheme in which ammonia was the first product to leave and aldehyde was released during the reoxidation process as the last product formed. The formation of aldehyde and the decolorization of the 470 nm chromophore on reduction of benzylamine oxidase with benzylamine could be conveniently studied by stopped-flow techniques. In Papers 4 and 5 the dependence of transient-state and steady-state reaction rate parameters on the concentrations of benzylamine and oxygen is examined.

The steady-state kinetic data confirmed that benzylamine oxidase operates by a ping-pong type of mechanism in which a substrate-reduced form of the enzyme appears as an intermediate in the catalytic process. At saturating concentrations of the amine substrate and physiological concentrations of oxygen, the reoxidation of the reduced enzyme by molecular oxygen is

rate-limiting under steady-state conditions. Reduction of the 470 nm chromophore under anaerobic conditions was found to correspond to a rate-limiting second-order interaction between enzyme and substrate. The second-order rate constant for decolorization of the 470 nm chromophore was quantitatively consistent with the steady-state kinetic parameters, indicating that the chromophoric changes induced by the substrate reflect the catalytically relevant reduction of the enzyme by substrate.

Benzaldehyde was found to be released in a pre-steady-state burst, the amplitude of which corresponded at saturating substrate concentrations to approximately one mole of product per mole of enzyme. This observation lends further support to the idea that benzylamine oxidase contains only one active site. The presence of this pre-steady-state burst of benzaldehyde formation, as well as the absence of any pronounced effect of oxygen concentration on the rate of product liberation, provides clear evidence that benzaldehyde is released prior to the rate-limiting interaction between oxygen and the reduced form of the enzyme. The benzaldehyde formation was found to be kinetically dependent upon a second-order interaction between enzyme and substrate which had a rate constant agreeing with that determined for the reduction of the 470 nm chromophore. Thus the two processes cannot be kinetically distinguished on the time-scale accessible to examination by stopped-flow techniques.

The kinetic data and other observations summarized here appear to be consistent with the following minimum reaction scheme:



This scheme is apparently inconsistent with the observation of Taylor et. al that no aldehyde was formed anaerobically. Preliminary experiments have suggested that hydrogen peroxide may also be released in a burst, and there is a possibility that all three reaction products (aldehyde, hydrogen peroxide and ammonia) could be formed prior to the rate limiting interaction between enzyme and oxygen. If so, the enzyme in its oxidized form must be expected to contain firmly bound oxygen. The observations made by Taylor et al. are therefore not necessarily incompatible with the present results; oxygen might dissociate from the enzyme at the extremely low oxygen concentrations reached in the experiments of these authors. At present stage of knowledge, the above simplified reaction scheme provides a satisfactory minimal model for the interpretation of kinetic data for benzylamine oxidase catalyzed reactions, although it is recognized that several additional enzymatic species of catalytic importance are undoubtedly formed in both reduction and reoxidation processes.

The results described in the present thesis have contributed to elucidation of the reaction mechanism of benzylamine oxidase, and have to some extent characterized the main functional roles of copper and pyridoxal phosphate in the protein. It is obvious, however, that much work remains to be done before the detailed sequence of reactions catalysed by the proteins that transfer electrons from the amine substrates to oxygen is known and understood.

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REFERENCES

1. Blaschko, H., Friedman, P.J., Hawes, R., Nilsson, N., J. Physiol., 145, 384 (1959).
2. Buffoni, F., Blaschko, H., Proc. roy. Soc. B 161, 153-167 (1964).
3. Blaschko, H., Buffoni, F., Proc. roy. Soc. B 163, 45-60 (1965).
4. Buffoni, F., Della Corte, L., Knowles, P.F., Biochem. J. 106, 575 (1968).
5. Lindström, A., Olsson, B., Pettersson, G., Eur. J. Biochem. in press. 47, 99-105 (1974).
6. Hamilton, G.A. (1968) in Pyridoxal Catalysis: Enzymes and Model Systems (Snell, E.E., Braunstein, A.E., Severin, E.S. and Torchinsky, Y.M., eds), 375-390, Interscience, New York.
7. Buffoni, F. (1968) in Pyridoxal Catalysis: Enzymes and Model Systems (Snell, E.E., Braunstein, A.E., Severin, E.S. and Torchinsky, Y.M., eds), 363-374, Interscience, New York.
8. Lindström, A., Olsson, B., Pettersson, G., Eur. J. Biochem. 42, 177-182 (1974).
9. Mondovi, B., Rotilio, G., Finazzi Agrò, A., Vallogini, M.P., Malmström, B.G., Antonini, E., FEBS Lett. 2, 182-184 (1969).
10. Kapeller-Adler, R., Amine oxidases and methods for their study, 124-134 (1970), Wiley, London.
11. Peisach, J., Levine, W.G., J. Biol. Chem. 240, 2284-2289 (1965).
12. Malmström, B.G., Reinhammar, B. and Vänngård, T., Biochim. Biophys. Acta 156, 67-76 (1967).

13. Andréasson, L.-E., Vänngård, T., *Biochim. Biophys. Acta* 200, 247-257 (1970).
14. Finazzi Agrò, A., Rotilio, G., Costa, M.T., Mondovi, B., *FEBS Lett.* 4, 31-32 (1969).
15. Taylor, C.E., Taylor, R.S., Rasmussen, C., Knowles, P.F., *Biochem. J.* 130, 713-728 (1972).
16. Mondovi, B. Finazzi Agrò, A. and Rotilio, G. in *Abstracts, 7th International Congress of Biochemistry, Tokyo, August 19-25, 1967*, 826, (1967).
17. Yamada, H. and Yasunobu, K.T., *J. Biol. Chem.* 237, 3077-3082, (1962).
18. McHenry, E.W. and Gavin, G., *Biochem. J.* 26, 1365-1377 (1932).
19. Kapeller-Adler, R. and McFarlane, H., *Biochim. Biophys. Acta* 67, 542-565 (1963).

